PB86-175387

Role of Glutathione Depletion in the Cytotoxicity of Acetaminophen in a Primary Culture System of Rat Hepatocytes

Procter and Gamble Co., Cincinnati, OH

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

1985

U.S. Department of Commerce
National Technical Information Service
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TECHNICAL REPORT DATA
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1. REPORT NO. EPA/600/J-85/343	2.
3. RI PB86-175387	
4. TITLE AND SUBTITLE ROLE OF GLUTATHIONE DEPLETION IN THE CYTOTOXICITY OF ACETAMINOPHEN IN A PRIMARY CULTURE SYSTEM OF RAT HEPATOCYTES (Journal Version)	5. REPORT DATE 1985
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) David B. Mitchell[1], Daniel Acosta[2], and James V. Bruckner[3]	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS [1]Miami Valley Labs, P&G, Cincinnati, OH 45247; [2]College of Pharm., Dept. of Pharm. & Toxicol., U. of Texas, Austin, TX 78712; & [3]College of Pharm. & Toxicol, U. of Georgia, Athens, GA 30602	10. PROGRAM ELEMENT NO. CBMC1A
	11. CONTRACT/GRANT NO. CR811215
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory (HERL) Office of Research and Development (ORD) U.S. Environmental Protection Agency (USEPA) Research Triangle Park, North Carolina 27711	13. TYPE OF REPORT AND PERIOD COVERED Journal
	14. SPONSORING AGENCY CODE EPA-600/11
15. SUPPLEMENTARY NOTES P.O. Paul T. McCauley, TOTB/TMD/HERL/USEPA (CI) Citation: Toxicology, 37 (1985) 127-146 October	

16. ABSTRACT

A primary culture system of postnatal rat hepatocytes was utilized to study the cytotoxicity of acetaminophen and the toxicological significance of glutathione (GSH) depletion. The relative time of onset and magnitude of GSH depletion, lipid peroxidation and cytotoxicity were contrasted in order to gain insight into their interrelationships. Exposure of the hepatocytes to acetaminophen resulted in time- and dose-dependent depletion of cellular GSH. The acetaminophen-induced GSH depletion and ensuing lactate dehydrogenase (LDH) leakage were quite modest and delayed in onset, in contrast to that caused by iodoacetamide (IAA) and by diethylmaleate (DEM), 2 well-known depletors of GSH. There was comparable LDH leakage, irrespective of drug treatment, when GSH levels decreased to about 20% of normal. Reduction of GSH levels below the 20% threshold by IAA treatment resulted in marked LDH leakage and loss of viability. Maximal LDH leakage in response to IAA and acetaminophen preceded maximal malondialdehyde (MDA) formation, suggesting that lipid peroxidation may be a consequence of cell damage as well as GSH depletion. IAA and DEM produced a comparable, modest accumulation of MDA, yet IAA was much more cytotoxic. These findings indicate that lipid peroxidation does not play a central role in hepatocellular injury by compounds which deplete GSH, although it may contribute to degeneration of the cell. As events in the cultured postnatal hepatocytes paralleled those reported in vivo, the system can be a useful and valid model with which to study mechanisms of chemical toxicity.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS *(This Report)* UNCLASSIFIED	21. NO. OF PAGES 21
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

EPA/600/J-85/343
JOURNAL ARTICLE

Toxicology, 37 (1985) 127–146
Elsevier Scientific Publishers Ireland Ltd.

ROLE OF GLUTATHIONE DEPLETION IN THE CYTOTOXICITY OF ACETAMINOPHEN IN A PRIMARY CULTURE SYSTEM OF RAT HEPATOCYTES*

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(Received December 31st, 1984)
(Accepted June 11th, 1985)

SUMMARY

A primary culture system of postnatal rat hepatocytes was utilized to study the cytotoxicity of acetaminophen and the toxicological significance of glutathione (GSH) depletion. The relative time of onset and magnitude of GSH depletion, lipid peroxidation and cytotoxicity were contrasted in order to gain insight into their interrelationships. Exposure of the hepatocytes to acetaminophen resulted in time- and dose-dependent depletion of cellular GSH. The acetaminophen-induced GSH depletion and ensuing lactate dehydrogenase (LDH) leakage were quite modest and delayed in onset, in contrast to that caused by iodoacetamide (IAA) and by diethylmaleate (DEM), 2 well-known depletors of GSH. There was comparable LDH leakage, irrespective of drug treatment, when GSH levels decreased to about 20% of normal. Reduction of GSH levels below the 20% threshold by IAA treatment resulted in marked LDH leakage and loss of viability. Maximal LDH leakage in response to IAA and acetaminophen preceded maximal malondialdehyde (MDA) formation, suggesting that lipid peroxidation may be a consequence of cell damage as well as GSH depletion. IAA and DEM produced a comparable, modest accumulation of MDA, yet IAA was much

*This work was supported in part by Cooperative Agreement CR 811215 with the U.S. Environmental Protection Agency. Presented in part at the annual meeting of the Society of Toxicology in Boston, MA, February, 1982.
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Abbreviations: DEM, diethylmaleate; DMSO, dimethylsulfoxide; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; GSH, glutathione; IAA, iodoacetamide; LDH, lactate dehydrogenase; MDA, malondialdehyde.

0300–483X/85/$03.30
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more cytotoxic. These findings indicate that lipid peroxidation does not play a central role in hepatocellular injury by compounds which deplete GSH, although it may contribute to degeneration of the cell. As events in the cultured postnatal hepatocytes paralleled those reported in vivo, the system can be a useful and valid model with which to study mechanisms of chemical toxicity.

Key words: Acetaminophen; Iodoacetamide; Diethylmaleate; Hepatotoxicity; Glutathione depletion; Lipid peroxidation; Cultured postnatal hepatocytes.

INTRODUCTION

As glutathione (GSH) is involved in maintenance of membrane structural integrity and in a variety of other processes in cells [1], a basic concept has evolved that GSH depletion in itself is not compatible with cell survival. It is widely recognized that GSH plays an important role in the metabolism of a number of xenobiotics [2]. Acetaminophen, for example, is chiefly metabolized in the liver by conjugation with glucuronic acid and sulfate, but a small portion is oxidized by the *P*-450 monooxygenase system to an electrophilic reactive intermediate which is conjugated by GSH [3,4]. Upon administration of high doses of acetaminophen to experimental animals, hepatic GSH levels diminish due to formation of an acetaminophen-GSH complex, and covalent protein binding of the metabolites and hepatocellular injury ensue. Thus, one of the most plausible mechanisms of acetaminophen-induced hepatotoxicity is that large doses of the drug deplete hepatic GSH. The reactive metabolite is then not completely detoxified and reacts with critical cellular macromolecules [5,6].

A second plausible mechanism for acetaminophen-induced hepatotoxicity involves GSH depletion and lipid peroxidation. As membrane systems in the cell contain large amounts of polyunsaturated fatty acids, lipid peroxidation can be a highly deleterious process. Lipid peroxidation is inhibited by hepatic GSH peroxidase, which utilizes GSH to reduce hydrogen peroxide and organic hydroperoxides, thereby preventing oxidative degradation of membrane lipids [7,8]. If GSH is depleted by acetaminophen, this protective process is compromised and lipid peroxidation and resulting cytotoxicity can occur [9].

Review of the published literature on acetaminophen hepatotoxicity reveals there is a lack of agreement on the mechanism of cellular injury by the drug. Investigators have recently employed in vitro systems in an attempt to understand events at the cellular and molecular levels. From studies in isolated rat hepatocytes, Anundi et al. [10] postulated that cellular damage following GSH depletion can be accounted for by lipid peroxidation, rather than protein binding. Harman and Fischer [11] found that acetaminophen

caused GSH depletion and lipid peroxidation in cultured hamster hepatocytes, but concluded that lipid peroxidation was more likely a consequence of cell damage rather than a causative factor. McLean and Nuttall [12] examined the toxicity of acetaminophen to rat liver slices, and theorized that the mechanism of cytotoxicity may involve processes subsequent to covalent binding and lipid peroxidation.

As cellular injury is complex, it is difficult to identify the initial key effect of a chemical and separate it from subsequent events which lead to cell death. It would appear that hepatocyte cultures may be a very useful tool for delineation of the sequence of events in cell injury. Hepatocytes can be maintained in culture for extended periods under carefully regulated, defined environmental conditions. It is possible to control and monitor precisely the concentration of test compound, and to record accurately the dynamic sequence of responses of the cultured cells to toxicants. We have developed primary cultures of postnatal rat hepatocytes which retain many of the differentiated functions of hepatocytes in vivo [13]. These functions can be monitored to insure continued functionality and uniformity of the cell populations, as well as to reveal injurious effects of cytotoxic chemicals. We have demonstrated that a wide variety of known hepatotoxins are toxic to the cultured postnatal rat hepatocytes in a dose- and time-dependent fashion [14–17]. A number of these chemicals including carbon tetrachloride, acetaminophen, papaverine and cyclophosphamide must be metabolically activated in the in vitro system in order to exert cytotoxicity. The system can be effectively utilized to rank the hepatotoxic potency of a series of congeners [18], as well as to distinguish between relatively toxic and nontoxic compounds [14,15].

In contrast to a number of other species, the rat has been shown to be resistant to acetaminophen hepatotoxicity [19,20]. Therefore, the cultured rat hepatocyte would appear to be an appropriate model system in which to investigate cellular protection mechanisms. Since GSH has been shown to play an important role in acetaminophen detoxification in vivo, a major objective of studies described in this paper was to relate GSH status to cellular integrity over time after the initiation of chemical exposure. Cellular responses to acetaminophen were contrasted with responses to 2 other compounds, diethylmaleate (DEM) and iodocetamide (IAA), which rapidly deplete GSH by dissimilar mechanisms. DEM is an unsaturated carbonyl compound which reduces liver GSH levels through a conjugation reaction catalyzed by GSH *S*-alkenetransferases [21,22]. The halogenated acetamide, IAA, directly alkylates GSH and other cellular macromolecules. The reaction of IAA with GSH apparently does not require enzyme catalysis [10,23]. A second major objective of the studies reported here was to delineate the relative time of onset and magnitude of GSH depletion, lipid peroxidation and cell membrane damage for each test compound, in order to clarify: (a) the relationship between GSH depletion and lipid peroxidation; and (b) the possible role of lipid peroxidation in cellular injury.

MATERIALS AND METHODS

Reagents

Culture medium and serum were purchased from the Grand Island Biological Co., Grand Island, NY. Plastic culture dishes were purchased from Falcon, Cockeysville, MD. Acetaminophen, iodoacetamide (IAA), maleic acid diethyl ester (diethylmaleate) (DEM), thiobarbituric acid, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and all other chemicals were purchased from the Sigma Chemical Co., St. Louis, MO.

Culture procedure

The isolation and primary culture of parenchymal hepatocytes from 10- to 11-day-old Sprague—Dawley rats has been described in detail elsewhere [24]. In brief, the procedure involved in situ perfusion of the liver with 0.05% (w/v) collagenase in Ca^{2+}-free Hanks' balanced salt solution to dissociate the tissue into individual cells. A suspension of about 5×10^6 cells was plated into individual 60 X 15-mm plastic culture dishes. The cells were cultured in Dulbecco-Vogt's modification of Eagle's minimum essential medium containing 0.1% (w/v) bovine serum albumin and 10% (v/v) newborn bovine serum supplemented with: hydrocortisone sodium succinate, 0.005% (w/v); insulin, 0.0005% (w/v); niacinamide, 0.03% (w/v).

The cultures were placed into a humidified environment of 5% CO_2—95% air and 37°C (Model CO-20 CO_2 Incubator, New Brunswick Scientific, New Brunswick, NJ). Culture medium, deficient in arginine but supplemented with 1×10^{-4} M ornithine, prevented the overgrowth of the parenchymal hepatocytes by fibroblasts and other non-parenchymal hepatocytes [25]. Thus, relatively pure cultures of parenchymal hepatocytes were maintained.

Drug treatments

Acetaminophen was dissolved directly in the culture medium to prepare concentrations ranging from 1×10^{-5} M to 5×10^{-3} M. IAA was also dissolved directly in the culture medium to prepare concentrations ranging from 1×10^{-6} M to 1×10^{-4} M. Three-milliliter portions of each solution were added to individual dishes of the 1-day-old cultures described above. Enough dimethylsulfoxide was added to an aliquot of DEM equal to 10X the final desired concentration to bring the total vol. up to 200 μl. This mixture was vortexed briefly, and 20 μl added directly to the culture medium. Three-milliliter portions of treated medium were then added to individual dishes as described above.

Glutathione assay

The GSH content of the parenchymal cell monolayer was estimated by the method initially described by Ellman [26] and later modified by Beutler et al. [27]. At the appropriate time-points after drug treatments, the culture medium was poured off and the culture dishes rinsed carefully with 0.3 M phosphate buffer. The cells were scraped off the dish with a rubber police-

man and sonicated in 2 ml buffer for 90 s. The resulting sonicate was centrifuged for 5 min (1500 *g*). Two milliliters of the supernatant were added to 3 ml of the protein precipitating solution (1.67 g metaphosphoric acid, 0.20 g EDTA, 30 g NaCl in 100 ml distilled water). The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at 200 *g*. Two milliliters of the resulting supernatant was added to 4 ml 0.3 M Na_2HPO_4 buffer and 1 ml of the Ellman reagent added (40 mg DTNB in 100 ml 1% sodium citrate). After mixing well, the solution was read at 412 nm in a Beckman Model 25 UV/VIS spectrophotometer. Absorbance values were compared to a standard curve generated from known GSH concentrations.

Assay for lipid peroxidation

The extent of lipid peroxidation was estimated by measuring malondialdehyde (MDA) production by the thiobarbituric acid reaction, as described by Buege and Aust [28]. Special culture medium had to be prepared that lacked phenol red, because this indicator interfered with the spectrophotometric readings.

Other assays

The total amount of protein per culture dish was determined by the method of Lowry et al. [29]. Leakage of the intracellular enzyme, lactate dehydrogenase (LDH), was measured in the medium as an index of cytotoxicity according to procedures previously described [30]. The viability of control and drug-treated cultures was assayed by the trypan blue dye exclusion method [31].

Statistical analysis

The statistical significance of differences in mean values of treated and control groups was assessed by analysis of variance, with $P < 0.05$ chosen as the minimum level of significance.

RESULTS

GSH depletion and toxicity caused by acetaminophen

The dose-dependent effects of acetaminophen treatment on GSH levels in 24-h-old parenchymal hepatocytes are shown in Fig. 1. As it has been noted previously that GSH levels in control cells slowly decline as a function of time in culture [32], the gradual decrement in GSH in untreated control cells is typical for 48-h-old cultures. All 4 doses of acetaminophen, ranging from 1×10^{-5} to 5×10^{-3} M, produce significant GSH depletion over the entire 24-h treatment period. The extent of GSH depletion is both concentration- and time-dependent. The majority of the decrease, however, occurs during the first 4 h of exposure. Maximal depletion of GSH does not exceed 50%.

The effect of acetaminophen treatment on cellular viability can be seen in Table I. Only the highest concentration of acetaminophen (5×10^{-3} M)

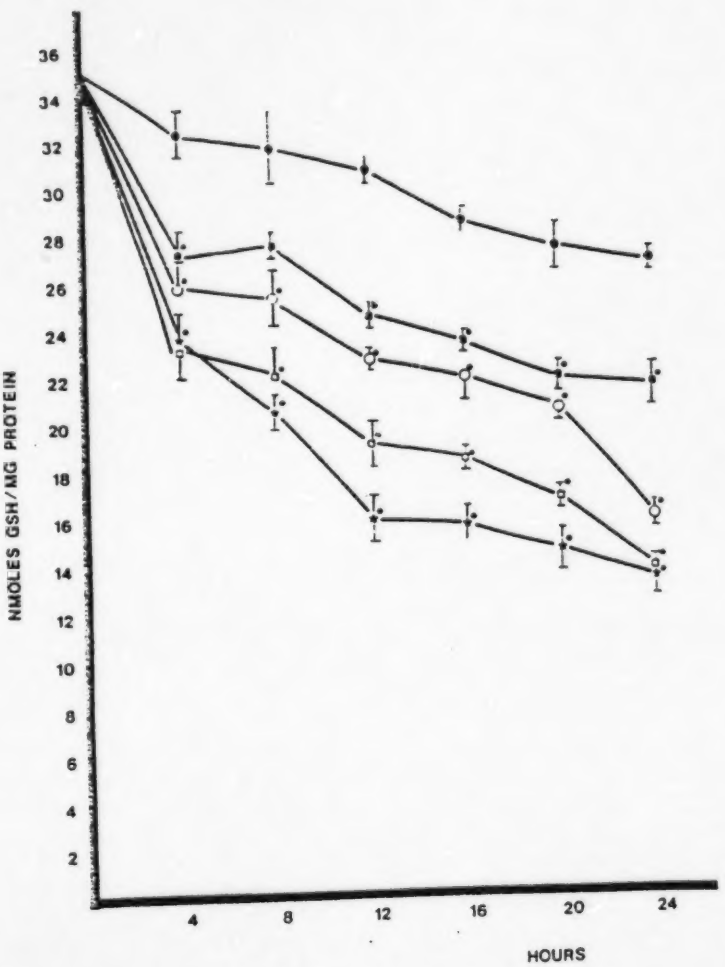

Fig. 1. Dose-dependent effects of acetaminophen on GSH levels in 24-h-old primary cultures of parenchymal hepatocytes. Five concentrations were employed: (•) none (control); (▪) 1×10^{-3} M; 1 (○) 1×10^{-4} M; (□) 1×10^{-3} M, (▪) 5×10^{-3} M. Each point represents the mean ± S.E. of 4 separate experiments. *Statistically different from controls, $P < 0.05$.

appears to affect viability. There is a slow, but progressive decrease in the viability of hepatocytes exposed to 5×10^{-3} M acetaminophen. This decrease is quite modest, in that only the 20- and 24-h values are significantly lower than controls.

Acetaminophen treatment also had a relatively modest effect on LDH

TABLE I

EFFECT OF ACETAMINOPHEN ON CELLULAR VIABILITY[a]

Exposure time (h)	Control	Acetaminophen concentration (M)			
		1×10^{-5}	1×10^{-4}	1×10^{-3}	5×10^{-3}
4	93.5 ± 0.6	92.5	91.5 ± 0.6	91.3 ± 0.3	90.5 ± 0.6
8	94.0 ± 0.4	93.0 ± 0.4	90.8 ± 0.5	90.8 ± 0.5	88.5 ± 0.6
12	92.0 ± 0.4	91.0 ± 0.4	89.0 ± 0.9	87.5 ± 0.9	85.8 ± 1.1
16	91.0 ± 0.4	91.3 ± 0.8	89.5 ± 0.6	88.8 ± 0.5	85.0 ± 1.8
20	91.0 ± 0.4	91.3 ± 0.9	88.8 ± 0.5	87.8 ± 0.5	84.3 ± 1.8[b]
24	91.5 ± 0.6	91.3 ± 0.8	88.8 ± 0.5	88.0 ± 0.4	84.0 ± 1.3[b]

[a] Each value (expressed as % viable cells) represents the mean ± S.E., $N = 4$.
[b] Statistically different from control, $P < 0.05$.

leakage (Table II). LDH leakage increased with increase in drug concentration above 1×10^{-4} M during the first 12 h of the experiment. 1×10^{-4} M and 1×10^{-5} M acetaminophen did not significantly enhance LDH leakage at most time points. There was no clear-cut time-course in LDH leakage. The magnitude of response after 4 h of exposure was comparable to that after 24 h.

The dose-dependent effects of acetaminophen treatment on lipid peroxidation in the cultured hepatocytes are shown in Fig. 2. Significant increases in the production of MDA were seen for each of the 3 doses of acetaminophen employed. The significant increases over controls were initially manifest at 8 h, at which time lipid peroxidation was maximal.

TABLE II

EFFECTS OF ACETAMINOPHEN ON LACTATE DEHYDROGENASE LEKAGE[a]

Exposure time (h)	Acetaminophen concentration (M)			
	1×10^{-5}	1×10^{-4}	1×10^{-3}	5×10^{-3}
4	107 ± 2	107 ± 2	112 ± 1[b]	121 ± 5[b]
8	108 ± 2	107 ± 2	112 ± 1[b]	121 ± 2[b]
12	106 ± 2	110 ± 4	114 ± 3[b]	122 ± 3[b]
16	112 ± 1	119 ± 4[b]	122 ± 3[b]	117 ± 1[b]
20	115 ± 1[b]	116 ± 3[b]	122 ± 4[b]	120 ± 4[b]
24	109 ± 2	108 ± 1	116 ± 4[b]	120 ± 3[b]

[a] Each value (expressed as % of control) represents the mean ± S.E., $N = 4$.
[b] Statistically different from control, $P < 0.05$.
Note: The % LDH data is expressed as % of T_0 control values. During a 24-h period of incubation, control cells leak approximately 60 units of LDH into the cell culture medium (T_0 = 81 units/ml/10^6 cells and T_{24} = 146 units/ml/10^6 cells). If the cells are totally lyzed, there is approximately 825 ± 8 units of LDH/10^6 cells.

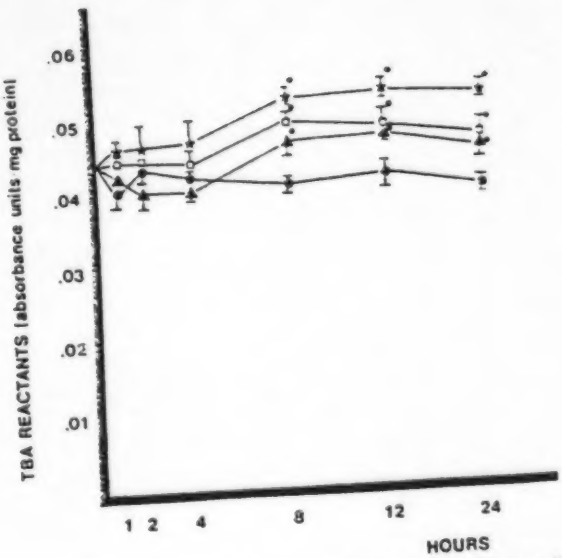

Fig. 2. Dose-dependent effects of acetaminophen on lipid peroxidation as measured by the thiobarbituric acid (TBA) reaction. MDA formation is expressed in terms of TBA reactants (absorbance units at 532 nm/mg protein). Four doses were employed: (•) none (control); (▲) 1×10^{-4} M; (○) 1×10^{-3} M; (▪) 5×10^{-3} M. Each point represents the mean ± S.E. of 4 separate experiments. *Statistically different from controls, $P < 0.05$.

GSH depletion and toxicity caused by IAA

IAA treatment produced a very dramatic and rapid depletion of cellular GSH levels within the first hour of exposure (Fig. 3). There was no additional decrease in GSH levels after 1 h at any exposure level. The depletion of GHS by IAA was concentration-dependent. 1×10^{-4} M IAA depleted GSH levels by more than 90%.

The dose-dependent effects of IAA treatment on LDH leakage are shown in Table III. The lowest concentration of IAA (1×10^{-6} M) produced a relatively minor increase in LDH leakage, and had relatively little effect on cellular GSH levels (Fig. 3). Exposure to 5×10^{-6} M and 1×10^{-5} M IAA resulted in comparable LDH release, and in 66% and 80% depletion of GSH, respectively. The 2 highest concentrations of IAA caused very marked LDH leakage (Table III) and significant decreases in cellular viability (data not shown). As illustrated in Fig. 3, 5×10^{-5} M and 1×10^{-4} M IAA also caused very marked GSH depletion (>90%). These doses were so toxic that they caused noticeable disruption of the continuous parenchymal cell monolayer in the culture dishes. Marked LDH leakage occurred during the first hour of exposure. Little additional increase in leakage was manifest thereafter (Table III).

Figure 5A illustrates the extent of lipid peroxidation caused by IAA treat-

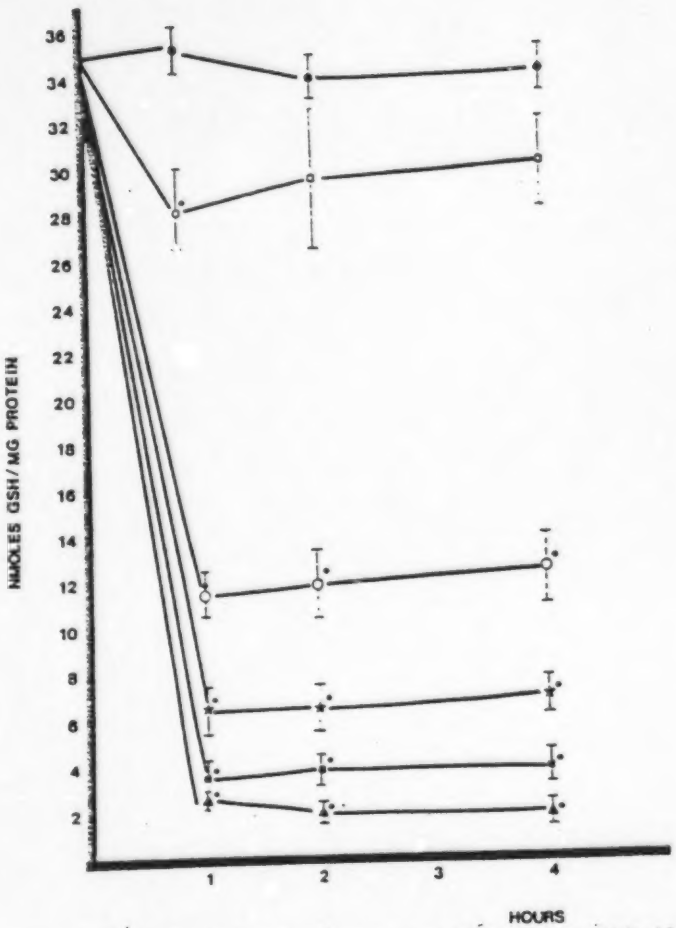

Fig. 3. Dose-dependent effects of IAA on GSH levels in 24-h-old primary cultures of parenchymal hepatocytes. Five concentrations were employed: (•) none (control); (▫) 1×10^{-6} M; (○) 5×10^{-6} M; (⋆) 1×10^{-5} M; (▲) 1×10^{-4} M; (■) 5×10^{-5} M. Each point represents the mean ± S.E. of 4 separate experiments. *Statistically different from controls, $P < 0.05$.

ment. The time intervals chosen for the assays ranged from 30 to 120 min, in order that they correspond to the period of rapid GSH depletion following IAA treatment. The lowest concentration of IAA (1×10^{-6} M) had relatively little effect at 60 min on MDA accumulation or on GSH levels (Fig. 3). MDA formation in response to 1×10^{-5} M IAA was time-dependent. The extent of lipid peroxidation caused by the highest concentration (1×10^{-4} M) of IAA was not time-dependent, but was already near maximal at 30 min.

TABLE III

DOSE-DEPENDENT EFFECTS OF IODOACETAMIDE ON LACTATE DEHYDROGENASE LEAKAGE[a,b]

Exposure time (h)	Iodoacetamide concentration (M)				
	1×10^{-4}	5×10^{-4}	1×10^{-3}	5×10^{-3}	1×10^{-2}
1	115 ± 2	164 ± 6	160 ± 4	272 ± 7	525 ± 26
2	125 ± 2	182 ± 12	173 ± 2	304 ± 19	647 ± 4
4	125 ± 1	170 ± 9	176 ± 11	463 ± 22	604 ± 36

[a] Each value (expressed as % of control) represents the mean ± S.E., $N = 4$.
[b] All values are statistically different from control, $P < 0.05$.

GSH depletion and toxicity caused by DEM

GSH depletion produced by DEM is illustrated in Fig. 4. DEM is a water insoluble liquid that was solubilized in the solvent dimethylsulfoxide (DMSO) prior to addition to the culture medium. DMSO alone had no effect on GSH levels in the cultured hepatocytes. Significant GSH depletion was achieved by all 6 doses of DEM employed, but the increment of depletion diminished as the DEM dose was increased. Doses of DEM greater than 2.0 μl/10 ml culture medium were not utilized because of solubility problems that yielded inconsistent results. The highest dose of DEM depleted GSH by about 80%, though this dose was 10 times greater (when calculated on a molar basis) than the dose of IAA required to deplete GSH levels by approximately 90%. GSH depletion was maximal within the first hour of treatment with DEM.

The dose-dependent effects of DEM treatment on LDH leakage are shown in Table IV. DMSO alone caused a slight increase in LDH leakage but had no effect on cellular viability (data not shown). The DEM data points were compared to the DMSO values, rather than the control values, in order to determine whether DEM significantly enhanced LDH leakage (Table IV). LDH leakage was maximal by 1 h at each dosage level. Significant decreases in cellular viability (data not shown) did not occur until the DEM dose reached 1.5 μl/10 ml culture medium. This DEM dose caused an approximate 75% decrease in GSH (Fig. 4). Although IAA was a more potent GSH depletor than was DEM, it was interesting to note that doses of the compounds which produced a comparable level of GSH depletion produced approximately the same degree of LDH leakage. For example, 1×10^{-5} M IAA and 2.0 μl DEM/10 ml culture medium each produced about 80% depletion of GSH and about a 60% increase in LDH leakage.

The influence of DEM treatment on lipid peroxidation is shown in Fig. 5B. The extent of MDA accumulation produced by each dose of DEM was maximal at 1 h. The effect was not dose-dependent over the entire range of

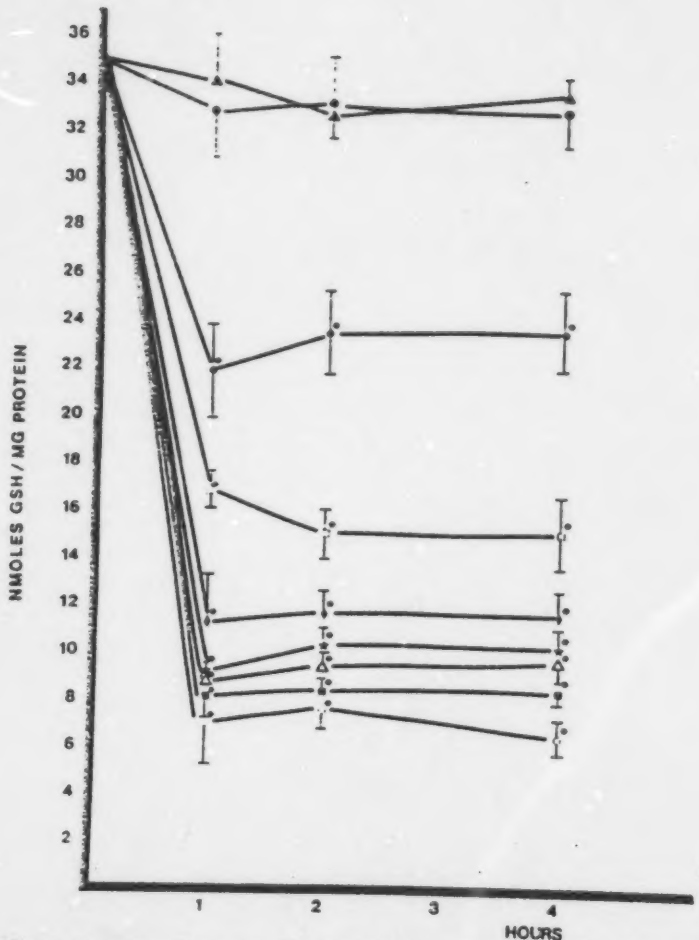

Fig. 4. Dose-dependent effects of DEM on GSH levels in 24-h-old primary cultures of prenchymal hepatocytes. Doses of DEM (expressed in terms of μl DEM/10 ml culture medium) were as follows: (•) none (control); (▲) DMSO vehicle only, (•) 0.1; (▫) 0.3; (◇) 0.5, (▪) 0.7, (△) 1.1, (■) 1.5, and (○) 2.0 μl/10 ml. Each point represents the mean ± S.E. of 4 separate experiments. *Statistically different from controls, $P < 0.05$.

doses utilized, in that the 2 highest concentrations (1.1 and 2.0 μl/10 ml medium) of DEM produced a similar degree of lipid peroxidation at each time-point. This effect was similar in magnitude to that caused by the highest dose of IAA (Fig. 5A).

GSH depletion and toxicity of treatment with acetaminophen and DEM

A series of experiments was designed to assess the effect on parenchymal

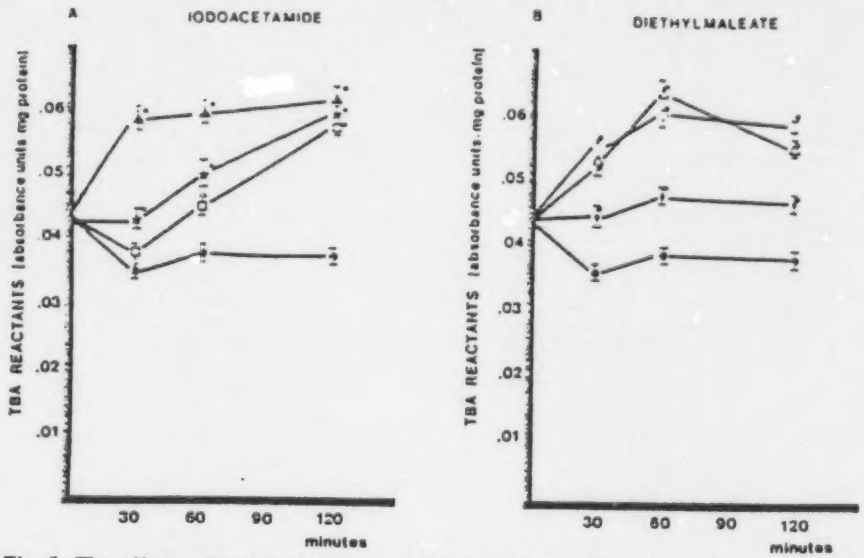

Fig. 5. The effects of IAA and DEM on lipid peroxidation. MDA formation is expressed in terms of TBA reactants (absorbance units at 532 nm/mg protein). A. *Iodoacetamide.* Four doses were employed: (•) none (control); (○) 1×10^{-4} M; (▪) 1×10^{-3}; and (▲) 1×10^{-2} M. B. *Diethylmaleate.* Four doses were employed: (•) none (control); (◇) 0.5, (△) 1.1, and (○) 2.0 µl/10 ml medium. Each point represents the mean ± S.E. of 4 separate experiments. *Statistically different from controls, $P < 0.05$.

TABLE IV

DOSE-DEPENDENT EFFECTS OF DIETHYLMALEATE (DEM) ON LACTATE DEHYDROGENASE LEAKAGE[a]

Dose	Time of exposure		
	1 h	2 h	4 h
DMSO	110 ± 2	112 ± 2	105 ± 3
0.1[b]	118 ± 6	119 ± 4	104 ± 2
0.3	121 ± 3[c]	122 ± 2[c]	108 ± 1
0.5	123 ± 4[c]	116 ± 6[c]	107 ± 1
0.7	122 ± 6[c]	114 ± 4[c]	123 ± 3[c]
1.1	126 ± 0.3[c]	128 ± 2[c]	132 ± 6[c]
1.5	142 ± 2[c]	142 ± 8[c]	129 ± 9[c]
2.0	165 ± 6[c]	178 ± 3[c]	167 ± 7[c]

[a] Data (expressed as % of control) represent the mean ± S.E., $N = 4$.
[b] µl DEM/10 ml culture medium.
[c] Statistically different from corresponding DMSO value, $P < 0.05$.

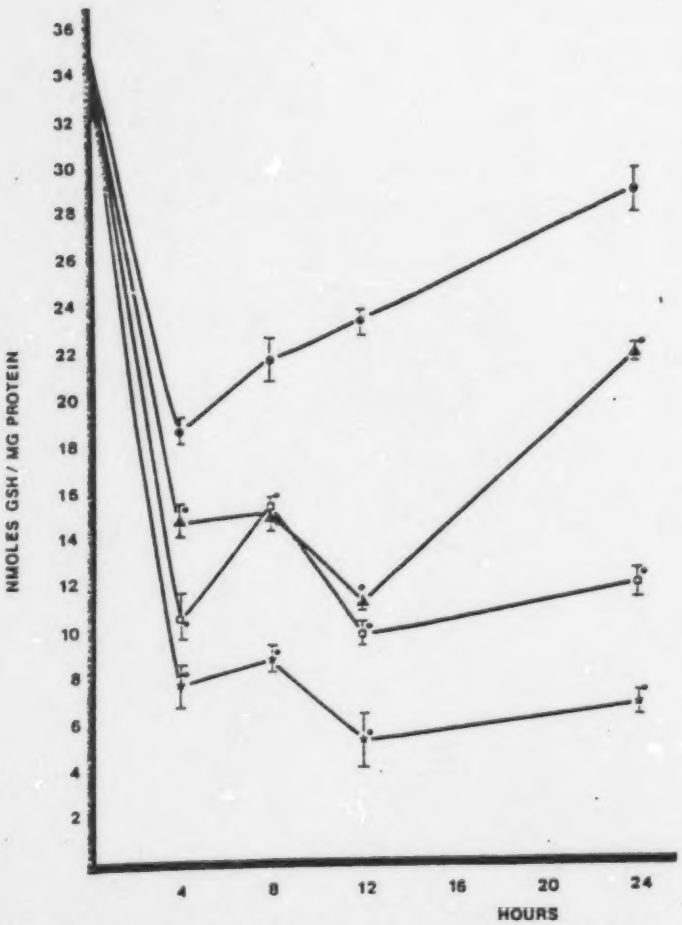

Fig. 6. Dose-dependent effects of concurrent acetaminophen and DEM treatment on GSH levels in 24-h-old primary cultures of parenchymal hepatocytes. Control culture groups received only 1.1 µl DEM/10 ml culture medium. Experimental cultures received 1.1 µl DEM/10 ml culture medium plus the designated acetaminophen dose. Four doses of ACMP were employed: (•) none (DEM controls); (▲) 1×10^{-4}; (○) 1×10^{-3} M and (*) 5×10^{-3} M. Each point represents the mean ± S.E. of 4 separate experiments. *Statistically different from controls, $P < 0.05$.

hepatocytes of concurrent administration of DEM and acetaminophen. A dose of 1.1 µl DEM/10 ml was chosen, because it caused significant GSH depletion by itself without resulting in marked toxicity. The doses of acetaminophen selected also had limited cytotoxic potential.

DEM, when administered with acetaminophen, produced greater GSH depletion (Fig. 6) than was caused by the same doses of acetaminophen

TABLE V

DOSE-DEPENDENT EFFECTS OF ACETAMINOPHEN ON LACTATE DEHYDROGENASE LEAKAGE IN CULTURES TREATED CONCURRENTLY WITH DIETHYLMALEATE[a,b,c]

Exposure time (h)	Acetaminophen concentration (M)		
	1×10^{-4}	1×10^{-3}	5×10^{-3}
4	138 ± 1[c]	145 ± 2[c]	151 ± 2[c]
8	130 ± 2[c]	137 ± 1[c]	144 ± 2[c]
12	128 ± 2[c]	132 ± 1[c]	136 ± 1[c]
24	118 ± 4[c]	134 ± 5[c]	156 ± 5[c]

[a] Each value (expressed as % of control) represents the mean ± S.E., $N = 4$.
[b] Control cultures were treated with 1.1 µl diethylmaleate/10 ml culture medium, experimental cultures were treated with 1.1 µl diethylmaleate/10 ml culture medium plus the designated acetaminophen dose.
[c] All values are statistically different from controls, $P < 0.05$.

alone (Fig. 1). GSH levels were reduced by approximately 80% by DEM and the highest dose of acetaminophen (5×10^{-3} M) (Fig. 6). GSH depletion was maximal in this experiment at 4 h, though substantial depletion may have been manifest sooner had earlier time-points been examined. Although GSH levels remained relatively low in hepatocytes subjected to DEM and the 2 highest concentrations of acetaminophen, there was recovery towards normal during the 24-h observation period in the cultures treated with DEM alone or DEM and 1×10^{-4} M acetaminophen. GSH levels in the cells treated with DEM alone returned to almost 100% of GSH levels in 48-h-old control cultures.

TABLE VI

EFFECTS OF CONCURRENT TREATMENT WITH ACETAMINOPHEN AND DIETHYLMALEATE (DEM) ON LIPID PEROXIDATION IN PRIMARY CULTURES OF RAT HEPATOCYTES[a,b,c]

Exposure time (h)	Control	Acetaminophen concentration (M)		
		1×10^{-4}	1×10^{-3}	5×10^{-3}
0	0.042 ± 0.003			
4	0.041 ± 0.004	0.047 ± 0.004	0.058 ± 0.003[c]	0.061 ± 0.002[c]
8	0.044 ± 0.003	0.050 ± 0.002	0.053 ± 0.002[c]	0.054 ± 0.002[c]
12	0.042 ± 0.002	0.047 ± 0.003	0.048 ± 0.003[c]	0.055 ± 0.004[c]
24	0.047 ± 0.002	0.053 ± 0.003	0.056 ± 0.002[c]	0.065 ± 0.003[c]

[a] Each value (expressed as absorbance units at 532 nm/mg protein) represents the mean ± S.E., $N = 4$.
[b] Control cultures were treated with 1.1 µl DEM/10 ml culture medium.
[c] Statistically different from controls, $P < 0.05$.

Treatment of the cultured hepatocytes with both DEM and acetaminophen resulted in LDH leakage, at 4 h (Table V), which was greater than that produced by either compound alone. Comparison of the 4-h values of the individual compounds (Tables II and IV) with the 4-h values of the compounds given in combination (Table V) revealed that the effects of DEM and acetaminophen on LDH leakage are additive. It can be seen in Table V that LDH leakage increases with increasing dose of acetaminophen. LDH leakage is maximal at 4 h at each dosage-level. It is worthy of note that the combined doses of DEM and acetaminophen, which produce 37.8, 44.8 and 50.8% increases in LDH leakage, also produce GSH depletion of approximately 60, 70 and 80%, respectively (Fig. 6).

The addition of DEM to culture medium containing acetaminophen resulted in greater formation of MDA than resulted from treatment of cultures with the same dose of acetaminophen alone (Table VI). The MDA levels, however, did not exceed levels produced by the same dose of DEM alone.

DISCUSSION

Acetaminophen exhibits limited capacity to deplete GSH and very limited cytotoxic potential under the experimental conditions employed in our rat hepatocyte culture system. This finding is in agreement with findings of Mitchell and his co-workers [19,20] in vivo, who observed that rats were resistant to acetaminophen hepatotoxicity. Ioannides et al. [33] found rats to be the least efficient of the species they studied in metabolically activating acetaminophen. Rats have also been found to be resistant to acetaminophen-induced GSH depletion [6]. In the present study GSH levels in cultured rat hepatocytes exposed to the highest concentration of acetaminophen (i.e., 5×10^{-3} M) diminished by just 30% during the initial 4 h of acetaminophen exposure. Harman and Fischer [11] recently reported that GSH levels in cultured hamster hepatocytes decreased by 80% after exposure for 4 h to 5×10^{-3} M acetaminophen. There was marked LDH leakage from the hamster hepatocytes, in contrast to the slight LDH leakage we measured from rat hepatocytes. This species difference has also been observed in vivo. Hamsters and mice given 300 mg acetaminophen/kg i.p. developed hepatic necrosis, but rats did not [20]. Hepatic GSH levels were reduced by 80% in the hamsters and mice, but only by 10% in the rats.

The results of our time-course and dose-response studies of acetaminophen support the concept that acetaminophen-induced GSH depletion may result in lipid peroxidation, but that the lipid peroxidation does not play a major role in acetaminophen cytotoxicity. Modest increases in MDA accumulation were first manifest at 8 h, by which time GSH levels had fallen approximately 40% (to 20—22 nmol/mg protein), in response to 1×10^{-3} M and 5×10^{-3} M acetaminophen. Wendel et al. [9] similarly saw relatively little evidence of lipid peroxidation in acetaminophen-dosed mice in which hepatic GSH levels were reduced by 50%. Mice with 90% GSH

depletion, however, experienced marked lipid peroxidation. Wendel et al. [9] calculated that the GSH threshold for prevention of lipid peroxidation was about 18 nmol GSH/mg protein. Harman and Fischer [13] monitored MDA formation in cultured hamster hepatocytes exposed to 5×10^{-3} M acetaminophen for up to 4 h. A 2-fold increase in MDA at 4 h was preceded by marked LDH leakage during the previous hour. Maximal LDH leakage in response to 5×10^{-3} M acetaminophen in the current study preceded the manifestation of significant MDA formation by 4 h. Although lipid peroxidation increased by 8 h and remained elevated during the 24-h study period, LDH values remained constant. These results suggest that lipid peroxidation may be the consequence of acetaminophen-induced cell damage rather than the cause of it. Carbon tetrachloride, a hepatotoxin thought to act primarily via lipid peroxidation, has been shown to cause lipid peroxidation in isolated rat hepatocytes prior to the onset of increased cell membrane permeability [34]. Smith et al. [34] saw a 10—20-fold increase in MDA production in isolated rat hepatocytes exposed to 5×10^{-3} M carbon tetrachloride for 4 h. This is far greater than the very modest degree of lipid peroxidation produced by acetaminophen in the current study.

We also examined the effects of other known GSH depletors in our hepatocyte culture system, in order to learn more of the relationships beween GSH depletion and lipid peroxidation. Addition of DEM to the culture media resulted in a rapid, dose-dependent fall in GSH levels, but in only a modest increase in lipid peroxidation. Wendel et al. [9] similarly found that i.p. injection of mice with doses of DEM, which markedly depleted hepatic GSH, had relatively little influence on lipid peroxidation. Younes and Siegers [35] did find significant MDA accumulation in livers from DEM-dosed rats, although it was necessary to pretreat the animals with phenobarbital to obtain this result. The need for an inducer, as well as the effectiveness of radical scavengers and monooxygenase inhibitors in preventing lipid peroxidation in liver homogenates, suggested to the investigators that the monooxygenase system itself has the potential to catalyze lipid peroxidation.

Results in the present study indicate that depletion of GSH in cultured hepatocytes to about 20% of normal is prerequisite for significant injury of the cells by the compounds studied. There was increased release of LDH in response to 1.5 and 2.0 μl DEM/10 ml media, concentrations which produced 77% and 80% GSH depletion, respectively. Interestingly, comparable LDH leakage occurred when GSH levels were depleted by 80%, irrespective of the treatment. Exposure of the hepatocytes to 2.0 μl DEM/10 ml media, to 1.1 μl DEM/10 ml media + 5×10^{-3} M acetaminophen, and to 1×10^{-5} M IAA all resulted in GSH depletion of approximately 80% and in LDH leakage that was 150—170% of controls. Higher concentrations of IAA (5×10^{-5} M and 1×10^{-4} M) reduced GSH levels by $\geq$90% and caused much more extensive cellular injury. Harman and Fischer [11] observed a similar phenomenon in cultured hamster hepatocytes. Exposure of these cells to concentrations of acetaminophen which depleted GSH by $\geq$80% resulted in

increasingly severe LDH leakage. It should also be noted in our own study and in that of Harman and Fischer [11] that GSH depletion preceded or accompanied the onset of loss of cell membrane integrity. A GSH depletion/hepatotoxicity threshold has also been seen in vivo. Hepatic necrosis was observed in mice and hamsters only in response to doses of acetaminophen which depleted hepatic GSH by 80—90% [5,6].

DEM appears to enhance the cytotoxicity of acetaminophen by acting in concert with the drug to reduce cellular GSH. It has been shown that DEM depletes hepatic GSH and potentiates the hepatotoxicity of acetaminophen in mice [5] and hamsters [6]. We found that concurrent exposure of cultured hepatocytes to DEM and acetaminophen resulted in greater GSH depletion than was produced by either compound alone. LDH leakage from these cells varied with the degree of GSH depletion. There was not the marked potentiation of cytotoxicity seen in vivo, however, because GSH levels were not reduced below the threshold of 80—90%.

It might be anticipated that liver cells depleted of GSH would subsequently restore their GSH levels through resynthesis. This process should occur over a period of hours after the GSH depleting agent has been removed, assuming the cells' synthetic mechanism is intact. Hepatic GSH levels in hamsters have been observed to return to and then to exceed control levels by 24 h after i.p. administration of 300 mg acetaminophen/kg body weight [6]. Harman and Fischer [11], however, saw no recovery of GSH levels in cultured hamster hepatocytes 12 h after the cells were initially exposed to 5×10^{-3} M acetaminophen. Similarly, we saw little evidence of GSH recovery within a 24-h period in cultured rat hepatocytes exposed to concentrations of acetaminophen as high as 5×10^{-3} M. This finding could be explained by a deficiency of GSH precursors (i.e., cysteine and methionine) in the culture media. Although we did not supplement the media with cysteine or methionine, sufficient amounts were apparently present for resynthesis to occur, since GSH levels returned towards normal over 24 h in hepatocytes treated only with DEM or DEM and 1×10^{-4} M acetaminophen (Fig. 6). Repletion of hepatic GSH following DEM administration has also been observed in vivo in mice and rats [36]. It can be seen in Fig. 6 that hepatocytes treated concurrently with DEM and acetaminophen exhibited a decreased ability to restore their GSH levels at the higher acetaminophen concentrations. It is not clear why GSH levels do not recover in cultured hepatocytes subjected to acetaminophen.

Our findings suggest that lipid peroxidation is not a major contributing factor to hepatocellular injury by compounds which deplete GSH. Maximal GSH depletion and LDH leakage are seen within 1 h of treatment of cultured hepatocytes with IAA. MDA levels, however, increase progressively for 2 h post-treatment. IAA and the 2 highest concentrations of DEM utilized produce a comparable increase in MDA formation, yet IAA is much more cytotoxic. The approximate 60% increase in MDA formation is far less than that produced in isolated hepatocytes by carbon tetrachloride [34], a chemical which causes peroxidative decomposition of membrane lipids. The cytotoxic

potency of IAA may be due to the compound's propensity to covalently bind to cellular macromolecules, once GSH is depleted. Anundi et al. [10] observed marked GSH depletion and protein binding within minutes after treatment of isolated rat hepatocytes with 5×10^{-5} M IAA. However these investigators concluded that hepatocellular damage was due to lipid peroxidation rather than protein alkylation, as they were able to inhibit the protein binding of IAA without affecting MDA accumulation or increased cell membrane permeability. Antioxidants were said to be effective in preventing cellular injury. Harman and Fischer [11], however, have discounted the role of lipid peroxidation in cellular injury by acetaminophen. They found no relationship between the abilities of monooxygenase inhibitors to prevent lipid peroxidation and to protect from cellular injury of cultured hamster hepatocytes subjected to acetaminophen. Recently, Devalia et al. [37] have also discounted the role of covalent binding in the cytotoxicity of acetaminophen. They were able to protect isolated rat hepatocytes from acetaminophen-induced damage without affecting protein covalent binding. Clearly, additional studies are required to clarify the sequence of events leading to the recovery or death of hepatocytes in acetaminophen poisoning. As events in the cultured postnatal rat hepatocytes parallel events in vivo, our culture system should be very useful in future mechanistic studies.

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